Genetics and Molecular Research

Association of the *GSTM1* null polymorphism with breast cancer in a Mexican population

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Genet. Mol. Res. 14 (4): 13066-13075 (2015)
Received May 15, 2015
Accepted July 31, 2015
Published October 26, 2015
DOI http://dx.doi.org/10.4238/2015.October.26.2

ABSTRACT. The glutathione S transferase (GST) family plays an important role in the processing of carcinogens. Data on the null *GSTM1* genotype has revealed associations with cancer, and has been suggested to affect carcinogen metabolism and to contribute to tumor promotion in

the mammary gland. We examined the role of the null *GSTM1* genotype by comparing the genotypes of 276 healthy Mexican women with those of 558 Mexican women with breast cancer (BC). The genotype frequencies observed in the controls and patients with BC were 38 and 45% for the null *GSTM1* genotype, respectively. The obtained odds ratio (OR) was 1.36, with a 95% confidence interval (95%CI) of 1.02-1.8, P = 0.04. The protective association was also evident upon analysis of the distributions of the null *GSTM1* genotype in patients with positive chemotherapy response who had high plasma levels of glucose (OR 0.56, 95%CI = 0.33-0.94, P = 0.03). This study suggested that the null *GSTM1* genotype is associated with BC susceptibility in the Mexican population analyzed.

Key words: *GSTM1* polymorphism; Breast cancer; Chemotherapy response; Mexican population

INTRODUCTION

Breast cancer (BC) is one of the most common diseases in developing countries and around the world, although the incidence rates of this disease vary in different ethnic groups (Miller et al., 2012; Siegel et al., 2012). In many countries, particularly in Mexico, the incidence of BC has increased in the last seven years, such that BC is now one of the main causes of death in working-age women (Chávarri-Guerra et al., 2012). The state of Jalisco exhibits one of the highest mortalities associated with BC, and only 10% of all cases of BC are detected early at stage I (Gómez Flores-Ramos et al., 2013; Ramírez-Patiño et al., 2013; Gallegos-Arreola et al, 2014). BC is considered to be a multifactorial disease and might result from a combination of abnormal gene interactions and environmental factors (Gómez Flores-Ramos et al., 2013; Gallegos-Arreola et al., 2014). Therefore, elucidating genetic variants among different ethnic groups could contribute to an explanation for the differences in the progression of cancer as well as in chemotherapeutic response between these groups.

Determination of the relationships between the detoxification of a wide variety of known and suspected carcinogens and the susceptibility to cancers including BC is a growing field of research (de Aguilar et al., 2012). The family of glutathione S-transferase (GST) cytosolic enzymes is involved in biotransformation phase II and acts on carcinogens, environmental pollutants, chemotherapeutic drugs, and other xenobiotics. This family is composed of six classes of dimeric isoenzymes (GST alpha, mu, pi, theta, zeta, and omega) that have been implicated as key detoxification enzymes. GST mu (GSTM1) is expressed in the liver, kidney, and lung and is involved in the detoxification of polycyclic aromatic hydrocarbons and other carcinogens. Individuals with the *GSTM1* null genotype are more susceptible to DNA damage than are wild type individuals (de Aguilar et al., 2012; Duggan et al., 2013). The *GSTM1* gene is located on chromosome 1p13.3, contains 10 exons, is polymorphic in humans, and possesses three known alleles: *GSTM1*A*, *GSTM1*B,* and *GSTM1*O* (or null). The *GSTM1 *A* (containing a lysine at position number 172 of the protein), and **B* (containing asparagine at this position) alleles differ only at a single amino acid (K172N) and appear to be functionally identical (McLellan et al., 1997).

The most common variant of *GSTM1* is the null genotype, which has been shown to be present in 20 to 50% of the population and lacks detectable expression of the gene product

(Duggan et al., 2013). This leads to a lack of expression of the GST mu enzymes that results in decreased antioxidant activity in the cell (Duggan et al., 2013), high accumulation of reactive oxygen species (ROS), and consequently higher susceptibility to carcinogenic events due to DNA damage (McLellan et al., 1997; Wang et al., 2010). It has been suggested that the null *GSTM1* is associated with an increased risk of developing some types of cancers including BC (McLellan et al., 1997; de Aguiar et al., 2012; Duggan et al., 2013; Possuelo et al., 2013; Wan et al., 2014). Wan et al. (2014), in a meta-analysis study, demonstrated an association of the null *GSTM1* genotype with BC in a Chinese population. Possuelo et al. (2013) found an association between the *GSTM1* null genotype with BC in a Brazilian population. Sohail et al. (2013) similarly demonstrated an increased susceptibility to BC in null *GSTM1* members of a Pakistani population.

Several reports in the general Mexican population have described a high frequency of the null *GSTM1* allele of approximately of 37-44%, depending on the tested region (Pérez-Morales et al. 2011; Martinez-Ramirez et al., 2013; Sandoval-Carrillo et al., 2014). However, there are no descriptions to date of an association of the null *GSTM1* polymorphism with BC in Mexican women.

MATERIAL AND METHODS

Sample collection and patient information

DNA was extracted from peripheral blood leucocytes from blood samples collected from 276 healthy women recruited as volunteer blood donors using standard protocols (Miller et al., 1988). These volunteers were not matched by age with the patient group. Blood samples were also collected from 558 patients with a clinical and histological diagnosis of BC between June 2010 and April 2014. All of the individuals included in this study were residents of the metropolitan area of Guadalajara. All samples were obtained after a written informed consent form was signed, which was previously approved by ethical committee 1301, Western National Medical Center, Mexican Institute of Social Security, Guadalajara, Jalisco, Mexico. This study was conducted respecting national and international ethical standards. Efforts were made to ensure that siblings of individuals who had already been sampled were excluded. Clinical and demographic data were obtained using written questionnaires. All patients were also interviewed to determine their occupational exposure and as well as use of pharmacological therapies. The BC patient database and the DNA samples have been examined for other polymorphisms (Gómez Flores-Ramos et al., 2013; Ramírez-Patiño et al., 2013; Gallegos et al, 2014).

Genotyping

A multiplex PCR method was used to detect the presence or absence of the *GSTM1* gene in the genomic DNA samples of the study groups. Determination of the null *GSTM1* polymorphism was performed using the following primers (exons 4-5): 5'-CTG CCC TAC TTG ATT GAT GGG-3' and 5'-CTG GAT TGT AGC AGA TCA TGC-3', and amplification of the internal control was performed using the following primers: CFRT - EX4 5'-AGT CAC CAA AGC AGT ACA GC-3' and 5'-GCT ATT CTC ATC TGC ATT CC-3' (Baranova et al., 1997). The polymerase chain reaction (PCR) amplifications were performed in a 15 µL total volume containing 0.2 mM dNTPs (Invitrogen, Carlsbad, CA, USA), 7.5 pmol primers, 2.5 mM $MgCl_2$, 2.5 U Taq polymerase (Invitrogen), and 50 ng genomic DNA. The PCR conditions were as follows: 94°C (4 min), followed by 35 cycles of

94°C (50 sec), 62°C (1 min) and 72°C (1 min), with a final extension at 72°C (7 min). Using this procedure, two fragments of 271 bp (indicating the presence of *GSTM1*, "no null") and 189 bp (internal control) were obtained. Genotypic discrimination was performed using 6% polyacrylamide gel (29:1) electrophoresis, followed by silver staining (Figure 1) (Sanguinetti et al., 1994).

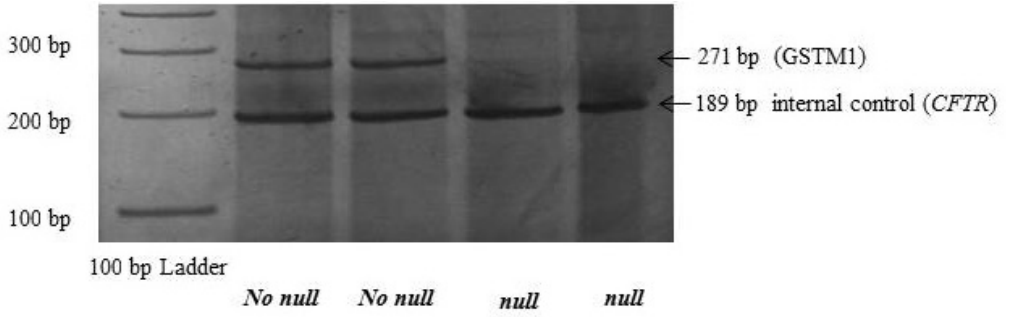

Figure 1. Polyacrylamide gel electrophoresis (6% (29:1)) detection of internal control (189 bp; *CFTR* exon 4) and *GSTM1* null (band absence) and "no null" (271 bp) genotypes.

Statistical analysis

Genotypic frequencies were obtained by direct counting and comparison by Chi square test among the study groups. Odds ratios and 95% confidence intervals (CIs) were also calculated. A two-sided $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using PASW Statistic Base 18 software, 2009 (Chicago, IL, USA).

RESULTS

The comparative demographic data from the patients with BC and the control individuals are shown in Table 1. In the patient group, the average age was 54.17 years, ranging from 25 to 88 years. Menarche presented at a mean age of 12.63 years in the patients and 12.09 years in the controls. Positive familial history of diabetes-arterial hypertension (DM2-AH) in first and second degree relatives of patients was observed to be risk factor (adjusted OR 1.8, 95%CI = 1.3-2.6, P = 0.001).

The clinical characteristics of the patients with BC included in the analysis were: body mass index (BMI: 18.5-24.9, ≥ 25-29.9, ≥ 30-34.9, and ≥ 35) (WHO Expert Consultation, 2004); tumor markers (luminal A, B, Her1/neu, and triple negative); histology (ductal, lobular); chemotherapy type (FEC-capecitabine: 5-fluorouracil-epirubicin-cyclophosphamide; others (paclitaxel, docetaxel, Herceptin); and no chemotherapy); and laboratory test: (hemoglobin, hematocrit (HTO), platelets, leukocytes, glutamate-oxaloacetate transaminase (SGOT), glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), and glucose). Table 2 presents the results of the multivariate logistic regression analysis from data included in the Table 1 and the clinical characteristics of BC listed above, where the BC group was classified using menopause status as the dependent variable. A lobular carcinoma (OR 2.3, 95%CI = 1.1-4.8, P = 0.022), presence of menarche (14-18 years) (OR 1.6, 95%CI = 1.03-2.5, P = 0.034), luminal B type (OR 1.9, 95%CI = 1.1-3.2, P = 0.017), and DM2-AH as personal medical history (OR 2.6, 95%CI = 1.6-4.3, P = 0.000) were found to be risk factors associated with menopause status.

Table 1. Epidemiological data of the study group.

	Patients with BC (N = 558)	Controls (N = 276)	OR (95%CI)*	P value
Age (years)				
Mean (SD)	54.17 (11.63)	35.31 (8.86)		<0.0001
Menarche (years)				
Mean (SD)	12.63 (1.67)	12.09 (0.82)		<0.0001
Menarche (range)				
7-10	(42) 8	(10) 4		
11-13	(350) 62	(248) 90		
14-18	(166) 30	(18) 6		
Tobacco consumption				
Yes	(149) 27	(61) 22		NS
No	(409) 73	(215) 78		
Alcohol consumption				
Yes	(105) 19	(52) 18		NS
No	(453) 81	(264) 82		
Family history (FH)				
No	(167) 28	(183) 66		
BC	(73) 13	(8) 3		
DM2-AH	(132) 24	(83) 30	1.8 (1.3-2.6)	0.001
DM2-AH-Cancer**	(196) 35	(2) 1		

BC = breast cancer; SD = standard deviation; NS = no significant difference; DM2-AH = diabetes mellitus type 2-arterial hypertension; OR = odds ratio; CI = confidence interval; *OR from the adjusted regression analysis; **positive familial history of cancer and leukemia in first and second degree relatives of patients.

The genotype frequencies of the null *GSTM1* allele were different in the control and patient groups (Table 3). The polymorphic genotype null *GSTM1* was observed in 45% (252/558) of the patients with BC and 38% (104/276) of the controls (OR 1.3, 95%CI = 1.02-1.8, P = 0.04). All of the samples were analyzed, and all of the participant genotypes (for 276 controls and 558 patients with BC) were obtained.

Table 2. Binary logistic regression analysis of the BC group.

	B	SD	Wald	d.f.	P	OR	95%CI	
							Lower	Upper
Lobular carcinoma	0.84	0.36	5.2	1	0.022	2.3	1.1	4.8
Menarche (14-18 years)	0.49	0.23	4.5	1	0.034	1.6	1.0	2.5
Luminal B	0.64	0.27	5.6	1	0.017	1.9	1.1	3.2
DM2-AH*	0.97	0.25	14.4	1	0.000	2.6	1.6	4.3

Variables included in the analysis: dependent: BC classified by menopause or pre-menopause status; independent: *personal medical history, menarche ranging from 7-10 years, 11-13 years, or 14-18 years of age; menopause, pregnancies, breastfeeding, oral contraceptive use, tobacco and alcohol consumption, a BMI of 18.5-24.9, ≥ 25-29.9, ≥30-34.9, or (obesity grade II-IV) ≥35-40, lymph node status, metastasis, response to chemotherapy, laboratory tests (hemoglobin (HB), hematocrit (HTO), platelets, leukocytes, urea, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and glucose). BC = breast cancer; SD = standard deviation; CI = confidence interval; OR = odds ratio; DM2-AH = diabetes mellitus type 2-arterial hypertension.

Table 4 demonstrates that the null *GSTM1* genotype was associated with high plasma levels of glucose (OR 0.62, 95%CI = 0.42-0.92, P = 0.018) and glutamic pyruvic transaminase enzyme (SGPT) (OR 0.50, 95%CI = 0.28-0.92, P = 0.027), and that the variables listed in Tables 1 and 2 were found to be protective factors.

Table 3. Genotype distributions of the *GSTM1* null polymorphism in patients with BC and healthy controls.

Genotypes	Patients (558)		Controls (276)		Patients *vs* Controls		
	(N)	%	(N)	%	OR	95%CI	P value
No null	(306)	55	(172)	62	1		
Null	(252)	45	(104)	38	1.3	(1.02-1.8)	0.0

BC = breast cancer; OR = odds ratio; CI = confidence interval.

Table 4. Association of the null genotype of the *GSTM1* gene with more than one variable among the general characteristics of the patients with BC.

	B	SD	Wald	d.f.	P	OR	95%CI	
							Lower	Upper
Glucose	-0.46	0.19	5.56	1	0.018	0.62	0.42	0.92
SGPT	-0.67	0.30	4.87	1	0.027	0.50	0.28	0.92
Constant	-0.00	0.10	0.002	1	0.963	0.99		

Variables included in the analysis: dependent: BC patients classified by W/ins-Ins/Ins genotype; independent: personal medical history, menarche ranging from 7-10 years, 11-13 years, or 14-18 years of age; menopause, pregnancies, breastfeeding, oral contraceptive use, tobacco and alcohol consumption, HF, HF type: BC, DM, AH, DM-AH-cancer, a BMI of 18.5-24.9, ≥25-29.9, ≥30-34.9, or ≥35->40, lymph node status, metastasis, response to chemotherapy, laboratory tests (Hemoglobin (HB), hematocrit (HTO), platelets, leukocytes, urea, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and glucose). BC = breast cancer; CI = confidence interval; SD = standard deviation; OR = odds ratio.

Additionally, the null *GSTM1* genotype was found to be a protective factor in chemotherapy response in patients with BC who had high plasma levels of glucose (OR 0.56, 95%CI = 0.33-0.94, P = 0.030, data not shown).

DISCUSSION

BC is a multifactorial disease with a complex etiology and is considered a major public health problem in industrialized countries. In Mexico, BC represents one of the leading causes of death in working-age women (Chávarri-Guerra et al. 2012; Miller et al., 2012; Gómez Flores et al., 2013; Ramírez-Patiño et al., 2013; Gallegos-Arreola et al., 2014). These facts are consistent with the observations made in the current study, where the average age of patients with BC was 54.17 (± 11.6) years. A familial history of DM2-AH was found to be risk factor for BC. Wolf et al. (2005) observed that DM2 is a serious health problem that affects more than 7% of adults in developed countries, that up to 16% of patients with BC have DM2, and that the two major risk factors for DM2, old age and obesity, are also associated with BC.

In our study, when the group was stratified by menopause stage as showing either pre-menopause or menopause, followed by comparison with the clinical and biochemical characteristics of BC, menarche (14-18 years), lobular carcinoma, luminal B type, and personal history of DM2-AH emerged as risk factors. It has been suggested that the effects of menarche and menopause on BC risk might be due to the function of endogenous ovarian hormones, that would be more relevant for estrogen receptor-positive disease than for estrogen receptor-negative disease and appear to be more relevant for lobular than for ductal tumors (Collaborative Group on Hormonal Factors

in Breast Cancer, 2012). Wolf et al. (2005) has postulated three mechanisms that could underlie the association of diabetes with BC: activation of the insulin pathway, activation of the insulin-like-growth-factor pathway, and regulation of endogenous sex hormones. Comparative cohort studies and case-control studies suggest that DM2 might be associated with a 10-20% excess relative risk of BC. Gestational diabetes mellitus, but not type 1 diabetes, might also be associated with an excess risk of BC. Furthermore, diabetes and its complications can adversely affect cancer therapy and the use of screening, which would affect the outcome of patients with BC.

The genetic regulation of phase II metabolism and its relation to carcinogenesis has been the focus of many investigations. GSTs are a family of important enzymes involved in phase II metabolism and the detoxification of a wide variety of known and suspected carcinogens, including potential mammary carcinogens (Possuelo et al., 2013; Wan et al., 2014). The null *GSTM1* polymorphism expresses no GSTM1 enzymatic activity and might present a decreased metabolism of carcinogens, which might accelerate neoplastic processes (Possuelo et al., 2013; Sohail et al., 2013; Wan et al., 2014). Therefore, studies to identify whether the null *GSTM1* polymorphism is associated with BC susceptibility have been performed in various populations (Possuelo et al., 2013; Sohail et al., 2013; Wan et al., 2014).

In the present study, we observed similar null *GSTM1* genotype frequencies in our control group at those that had been previously reported in Mexico (Pérez-Morales et al., 2011; Martínez-Ramírez et al., 2013; Sandoval-Carrillo et al., 2014). The genotypic *GSTM1* null frequencies were 38 and 45% in the control group and in patients with BC, respectively, suggesting an association of this allele as a risk factor in BC. Similarly, Wan et al. (2014) detected an association of the null *GSTM1* genotype with BC in a meta-analysis study of Chinese populations. Possuelo et al. (2013) also observed a higher frequency of the null *GSTM1* polymorphism in patients with BC compared with a control group in a Brazilian population.

In this study, we also observed a protective association of the null *GSTM1* genotype in patients with BC with high plasma levels SGPT and glucose. It is known that blood glucose levels are related to SGPT enzyme levels, because glucose is metabolized by the liver enzyme SGPT as an energy and amino acid source for the musculoskeletal system (Xiang et al., 2014). On the other hand, it has been observed that GSTs play an important protective role in preventing the elevation of SGOT and SGPT in the liver, and it is speculated that people with null *GSTM1* or *GSTT1* genotypes might not detoxify toxic reactive metabolites efficiently and thus might have a higher risk of elevated levels of SGOT and SGPT in the liver (Xiang et al., 2014). Other studies have been inconsistent about the relationship between the null *GSTM1* and *GSTT1* genotypes and high levels of SGOT and SGPT liver enzymes (Wang et al., 2010; Xiang et al., 2014). A plausible biological explanation for the protective association observed in this study might be that elevated glucose levels mimic the protective association of SGOT observed in patients with BC in this study. Alternatively, some studies have described an association of higher blood glucose levels with the incidence of BC (Wahdan-Alaswad et al., 2013; Xu et al., 2014).

Whereas glucose provides fuel for rapidly dividing cancer cells, insulin is a hormonal stimulator for cellular proliferation (Wahdan-Alaswad et al., 2013). It is also known that hyperglycemia is caused by non-internalization of glucose in the cells of diabetic patients, which contributes to increased ROS generation and oxidative stress which can damage the bases of DNA (Lazalde-Ramos et al., 2012). Sadi et al. (2013) observed that diabetes causes a significant decrease in the mRNA expression of *GSTM1*. It is known that the metabolic processes including glucose oxidation, enzymatic glycation of proteins, and subsequent oxidative degradation of glycated proteins can be

produce ROS in diabetic patients, and these changes subsequently reduce antioxidant defense mechanisms, simultaneously leading to lipid peroxidation and damage to cells. Thus, excessive oxidative stress occurring in the liver due to diabetes mellitus was suggested to result in the down-regulation of GST mu, superoxide dismutase, and catalase gene expression. Accordingly, decreased mRNA expression of the GST mu isoenzyme in patients with diabetes could be due to a decreased half-life of its mRNA because increased oxidative stress might lead to destabilization of mRNA (Sadi et al., 2013).

On other hand, in this study, we observed an association of the null *GSTM1* genotype as a protective factor for chemotherapy response in patients with BC who had high plasma levels of glucose. Soto-Quintana et al. (2011) observed a tendency toward better chemotherapy response in patients with the null genotype of *GSTT1* and *GSTM1* polymorphisms and advanced BC. Additionally, an improved survival in patients with BC carrying the null *GSTM1* genotype has been observed, and this has been explained by a better response to chemotherapeutic agents related to more effective cell killing, which in turn is related to the absence of a protective effect of the *GSTM1* allele (Lizard-Nacol et al, 1999). Tulsyan et al. (2013) observed a better pharmacogenetic influence of *GST* polymorphisms on anthracycline-based chemotherapy responses and toxicity in BC. Furthermore, Bai et al. (2012) found a significant association between *GSTM1* and *GSTP1* gene polymorphisms and clinical outcomes in patients with BC.

Therefore, it is thought that chemotherapy might have a better effect in patients with diabetes carrying the null *GSTM1* genotype; both cases might be due to the impossibility of eliminating (phase II) the secondary metabolites of chemotherapy. An increase in ROS production could lead to DNA damage in patients with diabetes who do not exhibit good metabolic control (high glucose); oxidative stress and elevated levels of glucose both reduce the activity of the family of GST enzymes. This effect combined with the null *GSTM1* genotype, which cannot affect metabolism of the chemotherapeutic agents, allows the drug to stay in the cell longer, producing a more efficient elimination of tumor cells.

Our results show that the frequencies of the null *GSTM1* genotype are significantly different in controls *vs* patients with BC. A protective association with chemotherapy response was also evident in patients showing high levels of glucose, which might contribute significantly to BC susceptibility in the analyzed sample from a Mexican population. Nevertheless, further studies are required to confirm or reject these conclusions.

ACKNOWLEDGMENTS

We thank nurses from [7]Highly Specialized Medical Unit of Gynecology-Obstetrics Hospital, Western National Medical Center, Mexican Institute of Social Security for facilitating sample collection and Western National Medical Center, Mexican Institute of Social Security for support of this project.

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